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(54) Title: CIRCULAR EXTENSION FOR GENERATING MULTIPLE NUCLEIC ACID COMPLEMENTS

(57) Abstract

A process for generating multiple linear complements of a single strand, circular nucleic acid template containing at least one cleavage site is described. The process consists of combining the single strand, circular nucleic acid template with polynucleotide primers under conditions sufficient for hybridization; extending the polynucleotide primer more than once around the circle to generate a complementary displacement of more than one contiguous complement of the single strand, circular nucleic acid template. Also described is a process of synthesizing novel single strand, circular nucleic acids between 30 and 2200 nucleotides. The process consists of synthesizing a linear polynucleotide; combining the linear polynucleotide with a complementary linking oligonucleotide under conditions sufficient for hybridization; and ligating the linear polynucleotide to produce a single strand, circular nucleic acid.

## CIRCULAR EXTENSION FOR GENERATING
## MULTIPLE NUCLEIC ACID COMPLEMENTS


### BACKGROUND OF THE INVENTION


This invention relates generally to the generation of
5 multiple complementary copies of a nucleic acid and, more
particularly, to the generation of such complementary
copies using novel circular templates.


Recombinant DNA techniques have revolutionized
molecular biology and genetics by permitting the isolation
10 and characterization of specific DNA fragments. Of major
impact has been the exponential amplification of small
amounts of DNA by a technique known as the polymerase chain
reaction (PCR). The sensitivity, speed and versatility of
PCR makes this technique amenable to a wide variety of
15 applications such as medical diagnostics, human genetics,
forensic science and other disciplines of the biological
sciences.


PCR is based on the enzymatic amplification of a DNA
sequence that is flanked by two oligonucleotide primers
20 which hybridize to opposite strands of the target sequence.
The primers are oriented with their 3' ends pointing
towards each other. Repeated cycles of heat denaturation
of the template, annealing of the primers to their
complementary sequences and extension of the annealed
25 primers with a DNA polymerase result in the amplification
of the segment defined by the 5' ends of the PCR primers.
Since the extension product of each primer can serve as a
template for the other primer, each cycle results in the
exponential accumulation of the specific target fragment,
30 up to several million fold in a few hours. The method can
be used with a complex template such as genomic DNA and can
amplify a single-copy gene contained therein. It is also
capable of amplifying a single molecule of target DNA in a

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complex mixture of RNAs or DNAs and can, under some
conditions, produce fragments up to ten kbp long. The PCR
technology is the subject matter of United States Patent
Nos. 4,683,195, 4,800,159, 4,754,065, and 4,683,202 all of
5   which are incorporated herein by reference.

While the possible applications of PCR are numerous,
they are limited to those situations where the DNA sequence
is known with sufficient specificity to design two primers
which hybridize to opposite strands of the target sequence.
10  Moreover, the repetitive cycles of heating and cooling,
primer hybridization and polymerization are time consuming
and optimally performed on expensive, automated
thermocycling devices.

Since the advent of PCR, several additional methods
15  have been devised to amplify the copy number of a target
sequence. Such methods employ a variety of strategies to
achieve the amplification of a small amount of target
sequence; however, all have inherent drawbacks.

The ligation chain reaction (LCR) is one such method.
20  See PCT publication WO 89/12696, which is incorporated
herein by reference. LCR uses a ligase enzyme to join
preformed stretches of DNA, termed "probes." Once these
probes find their complementary target sequences and anneal
to them, the ligase joins the target sequences together,
25  setting up a situation analogous to that found in PCR: two
templates are formed, both of which can serve in the next
cycle to generate more copies of the target sequence.

Qβ replicase is another alternative for exponentially
amplifying a target sequence. This method relies on the
30  ability of the enzyme Qβ replicase to generate copies of
recombinant RNA molecules. Qβ replicase is an RNA-directed
tRNA polymerase of bacteriophage Qβ. The sequence to be
amplified is cloned into a gene coding for a natural

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template of QB, such as MDV-1 RNA, and produced as a
recombinant RNA. For target amplification, the recombinant
RNAs hybridize to complementary DNA and serve as templates
for RNA synthesis. See Kramer and Lazardi, Nature 339:401-
5  402 (1989), which is incorporated herein by reference.

Transcription-based amplification (TAS) systems have
also been developed to amplify the copy number of a target
sequence, as described in PCT publication WO 88/10315. The
cycle begins with the synthesis of cDNA from a target RNA
10  sequence. TAS employs specialized 5' primers which contain
a RNA polymerase promoter sequence. The use of these
primers results in a promoter sequence being incorporated
into the double-stranded product. Transcription from the
promoter results in the production of multiple RNA copies.
15  These copies can then be reprimed for additional rounds of
cDNA synthesis and transcription. A disadvantage to this
method is that it requires accurate execution of at least
three enzymatic steps. Therefore, full length products may
be difficult to obtain.

20     Finally, enhancement of target sequences by priming,
extension, restriction and reannealing of a linear fragment
containing multiple copies of the target sequence has been
described. See Becker et al., EPA No. 0 300 796, which is
incorporated herein by reference. Although this method
25  generates additional copies of target sequence with each
cycle, it has a major disadvantage in that the template,
which consists of multiple copies of the target sequence,
is destroyed by restriction at the end of each cycle and
cannot be reused. Moreover, multiple copies must be
30  present in each template to provide any increase in copies,
and product transcripts are of highly variable length.

Most of the methods described above rely on thermal
cyclization for denaturing and reannealing of products
and templates. Methods such as the QB replicase technique

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additionally require specialized recombinant RNA templates. Moreover, all methods employ linear templates, some of which are specially constructed to contain repetitive copies of the sequence to be amplified.

5        There thus exists a need for a method to generate multiple copies of a target sequence without the use of degenerative or specialized linear templates and without time consuming thermal cyclizations. Such a method would be of critical importance to increasing the efficiency of
10      medical diagnosis. The present invention satisfies this need through the use of novel circular templates and provides related advantages as well.

## SUMMARY OF THE INVENTION

        The invention provides a process for generating
15      multiple linear complements of a single strand, circular nucleic acid template containing at least one cleavage site. The process includes combining the single strand, circular nucleic acid template with polynucleotide primers under conditions sufficient for hybridization; and
20      extending the polynucleotide primer more than once around the circle to generate a complementary displacement of more than one contiguous complement of the single strand, circular nucleic acid template. The invention further provides a process of synthesizing novel single strand,
25      circular nucleic acids between 30 and 2200 nucleotides, by synthesizing a linear polynucleotide and ligating the linear polynucleotide to produce a single strand, circular nucleic acid.

## BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 is a schematic diagram of a single-stranded
circle. A represents a target sequence, 10-1000 bases in
length, complementary to the polynucleotide primer; B and
5  C represent sequences, 0-400 bases in length, which are
used as detection sequences for circle complement; d-f
represent linking sequences of 0-100 bases in length.
Any of A-e may contain one or more restriction sites.

FIGURE 2 is a schematic diagram for generating
10  multiple nucleic acid complements. G represents single-
stranded circle primed for extension by a target
polynucleotide; H represents excess single-stranded circle;
I represents G extended by polymerizing enzymes after
displacement to form multiple linear concatemers; J
15  represents I cleaved into smaller fragments; K represents
H primed by fragments from J; Reaction 1 is extension of
primer by polymerizing enzymes; Reaction 2 is cleavage of
I to form J; Reaction 3 is reannealing of fragments from J
to circles H.

20  FIGURE 3 is a schematic diagram of constructs for
making single-stranded circles. L represents one linear
fragment circularized by linker m before ligation; N
represents formation of a circle composed of two or more
fragments o and p, each 15-1100 bases in length which may
25  be similar or different in sequence; Q represents formation
of a circle capable of forming at least one stem structure
by ligation of two subunits containing loops r and s; T
represents formation of a circle capable of forming at
least one stem structure by ligation of a single linear
30  fragment containing loops r and s; U represents formation
of a circle capable of forming one or more stem structures
with ends which can be ligated; V represents construction
of a circle by restricting a stem structure at site w,
allowing formation and ligation of the product circle.

## DETAILED DESCRIPTION OF THE INVENTION

This invention is directed to a simple, inexpensive
and rapid process for generating multiple linear
complements from a single strand, circular nucleic acid and
5  to a process for making such novel circular nucleic acids.
An important advantage of generating linear complements
from single strand, circular nucleic acids is that the need
for differential thermal conditions at each step in the
process is alleviated. All steps in the process can be
10  performed isothermally which also alleviates the need for
expensive thermocycling devices. Thus, the process is
applicable to use for detecting nucleic acids in such areas
as medical diagnosis and infectious diseases.

In one embodiment, the process for generating multiple
15  linear complements from single strand, circular nucleic
acid templates involves combining the template with
polynucleotide primers, such as an analyte, in a biological
sample. The combining step is performed under conditions
sufficient for hybridization so that a primer-template is
20  formed between the primer and a complementary region within
the circular template and can be used for polymerase
extension. The analyte can be, for example, of viral or
bacterial origin and may be DNA or RNA.

Extending the primer-template with a polymerase such
25  as Klenow fragment generates a linear concatemer of the
circular template through strand displacement synthesis.
The synthesized DNA segment is thereby replaced with a new
strand each time the polymerase passes around the circular
template to produce a complementary displacement product.

30      The circular template can contain a cleavage site such
as a restriction endonuclease cleavage site. The
restriction endonuclease site is incorporated into the
complementary displacement product and can be used, when

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hybridized to a restriction polynucleotide, to cleave the displacement product into unit length linear complements. The restriction polynucleotides are preferably the single strand, circular nucleic acid templates, wherein the
5  restriction site on the circular template has been modified so as to be refractory to endonuclease cleavage. Modification can be accomplished by methylation, for example.

The linear complements generated by strand
10 displacement synthesis and cleavage can be further used to prime additional circular templates for production of additional linear complements. All components necessary for polymerase extension and cleavage of the displacement product to linear complements remain available for use in
15 additional cycles. In this way, a large number of linear complements can be generated from a very small number of initial polynucleotide primers.

If the polynucleotide primers are, for example, analytes in a biological sample, then the production of
20 linear complements signifies the presence of the analyte. Detection of linear complements or displacement products can be performed by methods known to one skilled in the art. The invention, therefore, may be used for rapid and efficient detection of infectious diseases and diagnoses of
25 genetic disorders.

In another embodiment, the process for making novel circular nucleic acids for use in the process for generating linear complements involves the chemical synthesis of linear polynucleotides and their combination
30 with linking oligonucleotides. The linking oligonucleotides have sequences complementary to the ends of the linear polynucleotides and can be hybridized to the linear polynucleotides. Hybridization of the two types of molecules will bring the 5' and 3' ends of one or more

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linear polynucleotide together.  Ligating the ends of the
linear polynucleotides together produces a single strand,
circular nucleic acid.

      As used herein, the term "single strand, circular
5  nucleic acid template" or "circular template" refers to a
single strand nucleic acid polymer which has been joined
through its 5' and 3' ends to produce a circular molecule.
These single strand, circular templates include molecules
produced by methods disclosed herein for synthesis of small
10  single-stranded circles (between 30 and 750 nucleotides) as
well as molecules produced _in vivo_ or _in vitro_ through
bacteriophage replication mechanisms.  Circular molecules
produced entirely through chemical synthesis are included
as well.   The circular templates can be either
15  deoxyribonucleic or ribonucleic acids.

      As used herein, the term "cleavage site" refers to a
nucleotide sequence in which the phosphodiester backbone is
selectively broken.  For example, a nucleotide sequence
recognized by a restriction endonuclease is a cleavage site
20  because the enzyme will cut the phosphodiester backbone at
selective sites within the sequence.  Such cleavage sites
may be single or double-stranded, depending on the
endonuclease.  Also included are chemical cleavage sites
such as pyrimidine and purine cleavage reactions performed
25  in Maxam and Gilbert sequencing, or cleavage through
chemical methods such as oxidation as described in United
States Patent No. 4,795,700, which is incorporated herein
by reference.

      As used herein, the term "polynucleotide primer"
30  refers to any nucleic acid with a complementary sequence
sufficient to hybridize to the single strand, circular
template and be used as a substrate for polymerase
extension reactions.  This term includes an analyte within
a mixture of nucleic acids, synthesized oligonucleotides

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and linear complements produced from displacement products. RNA as well as DNA primers can be used. The length of the primer can vary so long as the 3' terminal nucleotides form sufficient base pairs to effectively prime the template.

5 Therefore, the term "primer-template" as used herein refers to a primer which is hybridized to a single strand, circular template and can be used in polymerase extension reactions.

As used herein, the term "displacement synthesis"
10 refers to polymerase extension reactions in which one strand of a double-stranded region is displaced by the processivity of the enzyme. Effectively, the strand is peeled away from the template simultaneously with new strand synthesis. Therefore, the term "complementary
15 displacement product" or "displacement product" as used herein refers to the displaced complementary strand which is peeled away from the template as the polymerase proceeds around the circular template.

As used herein, the term "restriction polynucleotide"
20 refers to a nucleic acid, preferably a single-stranded deoxyribonucleic acid, which encodes a restriction endonuclease site so that when hybridized to a complementary strand it forms a functional restriction endonuclease site. The length of the restriction
25 polynucleotides can vary so long as, when in duplex form, the appropriate restriction enzyme will recognize the sequence and cleave the phosphodiester bonds. Restriction polynucleotides may include linear poly- and oligo-nucleotides as well as circular nucleic acids such as the
30 circular templates described herein.

As used herein, the term "linear complement" refers to the complementary sequence of the single strand, circular template. A linear complement is the extension product from a primer-template substrate and includes concatemers

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produced by displacement synthesis as well as individual copies of the circle template.

As used herein, the term "blocked" refers to modifications of an oligo- or polynucleotide which limits
5 the function of the modified group. Such functions may include polymerization of a nucleotide or nucleotides. Blocking groups can include, for example, attachment of chemical moieties, organic polymers and macromolecules to the oligo- or polynucleotide. Also included is the reverse
10 addition of a nucleotide to the oligo- or polynucleotide. Modifications encompass additions to the nucleotide bases, the sugar moieties and the phosphate backbone.

The invention provides a process for generating multiple linear complements of a single strand, circular
15 nucleic acid template containing at least one cleavage site by combining the single strand, circular nucleic acid template with polynucleotide primers under conditions sufficient for hybridization; extending the polynucleotide primer more than once around the circle to generate a
20 complementary displacement product comprising more than one contiguous complement of the single-stranded, circular nucleic acid template; and cleaving the displacement product to generate linear complements of the circle template.

25 As shown in Figure 2(G), single strand, circular templates are first hybridized to polynucleotide primers. The resultant primer-templates are substrates for polymerase directed synthesis of nucleic acids. For example, if the template is DNA, then any of the known DNA
30 polymerases such as E. coli DNA polymerase I, Klenow fragment, T4 or T7 DNA polymerases, modified polymerases, reverse transcriptase, Taq polymerase, Bst polymerase, and T. flavins polymerase can be used to synthesize a complementary DNA strand. Alternatively, if the template

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is RNA, reverse transcriptase can be used to synthesize a complementary DNA strand. The invention provides a process for generating multiple copies of a single strand, circular nucleic acid template using the above polymerases.

5        The invention provides for polynucleotide primers which are selected so as to allow strand displacement synthesis by a polymerase. This displacement can be accomplished in a variety of ways. For example, primers can be chosen which are complementary over their entire
10   sequence to the circular template. Extension of the primer around the template will result in displacement of the 5' end of the primer by a polymerase. The resultant displacement product is a linear concatemer which is complementary to the circle template as shown in Figure
15   2(I). If a completely complementary primer is used, then the polymerase selected should preferably be devoid of 5' to 3' exonuclease activity, such as Klenow fragment. This selection prevents nick translation activity and ensures displacement synthesis.

20        Alternatively, polynucleotide primers can be selected in which the 5' end is non-complementary to sequences contained in the circle template, but the 3' end is complementary to contained sequences and hybridizes to the circle template. Extension of this primer-template around
25   the circle results in displacement synthesis at the juncture of the hybridized and non-hybridized regions of primer.

        Polynucleotide primers can also be selected wherein the 5' end of the nucleotide primers are blocked. Blocking
30   of the 5' end of a primer, as defined above, allows for displacement synthesis. As with the 5' non-complementary primers, a primer modified at its 5' end by, for example, a chemical moiety, will not impede a polymerase's movement, nor will it be recognized by inherent 5' to 3' exonuclease

activity found in most polymerases. Therefore, the invention provides a process for generating multiple complements of a circular template wherein the 5' end of the nucleotide primers are non-complementary to the
5   circular template or are blocked so as to allow displacement synthesis by a polymerase.

The invention further provides a process of synthesizing single strand, circular nucleic acids containing between 30 and 750 nucleotides by chemically
10  synthesizing a linear polynucleotide; combining the linear polynucleotide with a complementary linking oligonucleotide under conditions sufficient for hybridization; and ligating the linear polynucleotide to produce a single strand, circular nucleic acid. Ligation can be by enzymatic or
15  chemical means.

The linear polynucleotides are chemically synthesized by methods known to those skilled in the art (e.g., United States Patent No. 4,500,707, which is incorporated herein by reference). Current chemical synthesis methods may be
20  used to synthesize linear polynucleotides of up to about 200 bases in length.

To generate a single strand, circular nucleic acid of about 200 nucleotides, a linear polynucleotide of about 200 nucleotides is first synthesized. The 5' and 3' ends of
25  the linear molecules are brought together to form circular molecules by hybridization to complementary linking oligonucleotides Figure 3(L). The linking oligonucleotides are designed such that the 5' end of the linking oligonucleotide is complementary to the 3' end of
30  the synthesized linear polynucleotide and the remaining 3' end of the linking oligonucleotide is complementary to the 5' end of the linear polynucleotide. The ends of the linear polynucleotide which have been brought together in this way are covalently joined by ligation. Alternatively,

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the use of RNA ligase without the use of linking
oligonucleotides may be used to ligate a circle since RNA
ligase is able to ligate DNA ends together. Additionally,
larger single strand, circular nucleic acids composed of
5   multiple linear polynucleotides of the same sequence may be
achieved by varying the concentration of components in the
ligation.

The invention also provides for the synthesis of
single strand, circular nucleic acids, where the circular
10  nucleic acid is synthesized from two or more linear
polynucleotides Figure 3(N). Synthesis of larger, single
strand, circular nucleic acid templates can be
accomplished, for example, by synthesizing two halves of
the circle as linear polynucleotides. For example, to
15  generate a 400 nucleotide circle, two shorter linear
molecules whose sum is 400 nucleotides are first
synthesized.

The linear molecules are joined together to create a
circular molecule in the same fashion as that described
20  above. Complementary linking oligonucleotides are designed
to bring the 5' end of one linear molecule and the 3' end
of the second linear molecule together. For example, to
bring together two linear molecules which contain different
terminal sequences at each of the four ends, two linking
25  oligonucleotides are needed. One should be complementary
to the 5' end of the first linear molecule and to the 3'
end of the second molecule. The second linking
oligonucleotide should therefore be complementary to the 3'
end of the first molecule and to the 5' end of the second.
30  If the 5' and 3' ends of one linear molecule are identical
or similiar in sequence to the 5' and 3' ends, respectively
of the second linear molecule, then only a single
complementary oligonucleotide may be used. Hybridization
of the complementary oligonucleotides to the linear
35  molecules will bring together the four ends of the

molecules to form a non-covalently closed circle. The
phosphodiester backbone can be ligated together to form
covalently closed, single-stranded circles. In a similar
manner, circles composed of three or more linear
5   polynucleotide precursors can be produced. Additionally,
alternative strategies for ligation of linear
polynucleotides into circles may be employed and are shown
in Figure 3. Therefore, the invention also provides for
novel single strand, circular nucleic acid templates,
10  wherein the template comprises between 30 and 750
nucleotides and is made from chemically synthesized oligo-
or polynucleotides.

The single strand, circular nucleic acid templates of
the present invention can also be produced by a variety of
15  other methods well known to those skilled in the art. For
example, single strand circular templates can be derived
through _in vivo_ bacteriophage replication as described in
Sambrook et al., Molecular Cloning: A Laboratory Manual,
Cold Spring Harbor Laboratory, (1989); Ausubel et al.,
20  Current Protocols in Molecular Biology, John Wiley and
Sons, (1987); and Short et al., Nucleic Acids Research
16:7583-7600 (1988), all of which are incorporated herein
by reference. Alternatively, _in vitro_ replication methods
can be employed such as those described by Shavitt and
25  Liuneh, J., Bacteriology, 171:3530-3538 (1989), which is
incorporated herein by reference. The sequence to be
amplified can be cloned into a variety of available phage
vectors, such as pBluescript™ (Stratagene Cloning Systems,
La Jolla, CA) or vectors can be constructed such that only
30  pertinent sequences remain. The invention provides a
process to generate multiple complements of a circular
template, wherein the single strand, circular nucleic acid
template is derived from phage replication.

Alternatively, circular nucleic acid templates can be
35  generated by _in vitro_ polymerase extension reactions. For

example, sequences of preferably between 50 and 2200
nucleotides to be transformed into a circular template are
first synthesized as a double-s anded duplex by priming a
linear template and polymerase extension. Defined ends of
5    linear duplex can be produced by digesting with restriction
endonucleases known to cut at preselected sequences or by
using preselected primers in polymerase chain reaction
synthesis.    The linear extension products can be
circularized as a single-stranded molecule by first
10   denaturing the duplex and hybridizing it to a
oligonucleotide complementary to each end of the linear
template. Treatment of this structure with ligase results
in a single strand, circular template. Any linear
molecules can be digested with DNases (such as Exo VII) to
15   remove any residual linear fragments, including the
complementary strand which does not ligate. Therefore, the
invention provides a process to generate multiple
complements of a circular template wherein the circular
template is between 50 and 2200 nucleotides. Also provided
20   are novel single strand, circular nucleic acid templates
wherein the template is between 50 and 220 nucleotides.

Synthesis of circular nucleic acids can be
accomplished using a combination of the methods described
above as well as in conjunction with methods known in the
25   art.    For example, methodology using bifunctional
phosphorylating reagents as described by Capobianco et al.,
Nucleic Acids Research 18:2661-2669 (1990), which is
incorporated herein by reference, can be employed for the
synthesis of circular templates. This methodology is
30   amenable to synthesis and circularization of nucleotides
longer than four bases in length or can be used in
conjunction with conventional phosphoramidite methodology
to cyclize longer molecules such as between 50 and 750
nucleotides in length for chemically synthesized linear
35   polynucleotides. Thus, the invention additionally provides
a process to generate multiple copies of a single strand,

circular template, wherein the template is chemically synthesized and is between 30 and 750 nucleotides.

In another aspect, the invention provides methods of characterizing small single-stranded circles 50-2200 bases
5  in length, as are produced by any of the above methods. Circularization of linear fragments produces nucleic acid products which migrate as bands with shifted electrophoretic mobilities, usually slower than the linear fragment. Such circles can be distinguished from the
10  initial linear fragment(s) or from other linear by-products as follows: 1) by mobility shift relative to markers; 2) circles do not enzymatically degrade when treated with Exo VII (which requires free 3' or 5' ends); 3) circles can be primed and primers can be extended past the site of
15  ligation; and 4) when annealed with unique restriction polynucleotides at a site other than the ligation site and restricted with the appropriate endonuclease, circles form only one full-length linear fragment. Any non-circular fragments, including the linkers used in producing the
20  circle, will be degraded by Exo VII, cannot extend past the linear terminus and will produce at least two fragments when restricted.

The invention provides for a single strand, circular template which has at least one cleavage site. The
25  cleavage sites are used, for example, to reduce the concatemeric displacement product down to individual linear complements. These individual complements correspond to a whole or partial single copy of the circle template. Hybridization of a restriction polynucleotide or a single-
30  stranded circle to the complementary displacement product produces a local duplex region which is the length of the restriction primer. The duplexed region is a substrate for endonucleolytic cleavage when treated with the appropriate endonuclease (i.e., the endonuclease which recognizes the
35  encoded restriction site). Since the cleavage site is

encoded at a predetermined location, restriction of the site produces linear complements of defined length and sequence.

The invention also provides for a process to generate
5 multiple complements of a single strand, circular template where the template has a restriction endonuclease site which is capable of being modified so as to inhibit restriction when hybridized in duplex form. For example, if the restriction endonuclease activity can be blocked
10 with modified bases, then the appropriate restriction endonuclease will not cleave the DNA when one or both of the strands are modified. For example, endonucleases such as Hpa II, Hae II, Hae III and BstU1 are known not to restrict at methylated sites. A particular use for this
15 embodiment is depicted in Figure 2(J). Restriction of the displacement product down to individual linear complements as described above will also restrict the circle template to a linear form unless the site on the template is refractory to digestion. For example, modification of a
20 Hpa II site on the circle template by methylation as described by Nelson and McClelland, Nucleic Acids Research (1987), 15:219-230, and incorporated herein by reference, inhibits digestion of the hemi-methylated DNA and therefore preserves the structure of the circular template.
25 Preferably, the circular templates are methylated prior to initial hybridization with the polynucleotide primers by incorporation of modified bases such as 5-methylcytosine or methyladenosine into the linear polynucleotide during chemical synthesis as described by Ono and Ueda, Nucleic
30 Acids Res. (1987) 15:219-232, which is incorporated herein by reference. The circle can also be methylated by a variety of other methods known to one skilled in the art. Such methods include, for example, the use of specific methylase enzymes, such as Hpa II methylase, to methylate
35 sites in the circle. Therefore, the invention additionally provides a process to generate multiple complements of a

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circular template wherein the restriction enzyme site on the template is modified by methylation. When it is not known if restriction enzyme activity can be blocked, simple procedures using oligonucleotide duplexes in solution can
5  easily ascertain how much enzyme activity is reduced by site modification.

Restriction of the displacement product can be accomplished subsequent to, or preferably be performed simultaneously with, primer extension and displacement
10 synthesis. In the former case, restriction endonucleases can be added at a later time. Displacement synthesis can even be stopped, for example, by heat denaturation of the polymerase or by organic extraction of the reaction. Restriction polynucleotides are hybridized to the
15 displacement product followed by digested with the appropriate restriction endonuclease. In the latter case, restriction polynucleotides and restriction endonucleases are included in the extension reactions. As the displacement product is produced, the restriction
20 polynucleotides hybridize to the product and are concomitantly digested by the endonuclease (shown in Figure 2(J)).

During simultaneous synthesis and restriction, if extension products resulting from primer extension of the
25 hybridized restriction polynucleotides are undesirable, then restriction polynucleotides can be selected which cannot be used as substrates by a polymerase. These polynucleotides can be either the circular templates described herein which do not have free 3' terminal
30 nucleotides for polymerization or linear molecules which are not substrates for polymerization. For example, polymerase recognition of a primer-template requires that about three terminal nucleotides of the primer be correctly hybridized to the template (Kornberg, A., DNA REPLICATION,
35 W.H. Freeman and Company (1980), which is incorporated

herein by reference). Therefore, the use of linear restriction polynucleotides which have non-complementary 3' terminal nucleotides will inefficiently promote polymerase extension reactions. Alternatively, the 3' end of the 5 restriction polynucleotide can be chemically blocked to prevent polymerase extension. Addition of a blocking group such as chemical modification using a 2',3'-didedoxy nucleoside at the 3' end, reversing the orientation of the terminal nucleotide or covalently linking a non-hydroxy 10 moiety such as an alkylamine will also allow the simultaneous synthesis and restriction of displacement product without resulting in extension products off of the restriction polynucleotides. Thus, the invention provides a process to generate multiple complements of a circular 15 template where the restriction polynucleotides are deoxyribonucleic acids and the 3' end is inhibitory to primer extension by a polymerase.

The restriction polynucleotides can be further selected so that, once restricted, a fragment of the 20 restriction polynucleotide remains hybridized to the 5'- end of the displacement product. The length of the end of the polynucleotide from the restriction site necessary to retain hybridization under various conditions and temperatures once restricted is known to one skilled in the 25 art, or can be determined without undue experimentation. These procedures are described in detail in Ausubel et al., ibid. Restriction polynucleotides selected in this way allow the linear complements produced after digestion to be used either directly or indirectly as a polynucleotide 30 primer for further strand displacement synthesis. The hybridized portion of the restriction polynucleotide to the 5' end of the linear complement functions in an analogous fashion as the non-complementary or blocked polynucleotide primers described above and therefore allows further 35 hybridization to circular templates and polymerization.

The restriction polynucleotides can be further selected so that, once restricted, the fragment hybridized to the 3' end of the linear complement is unstable and does not remain hybridized. This restriction generates a
5   single-stranded 3' end of the linear complement which is available to be recognized as a polymerase substrate when hybridized to a circular template. Again, polynucleotide lengths which are unstable under various conditions and temperatures are known or can be determined by one of
10  ordinary skill in the art. For example, at 37°C, which is the optimal temperature for a polymerase, a restriction primer which results in hybridization of only about four nucleotides once it is digested will be unstable. Additionally, if modified circular templates are used as
15  restriction primers, the digested 3' end of the linear complements are already hybridized to the circular templates and can be extended without rehybridization to a circular template. The use of circular templates in this way is advantageous if restriction of the circle is
20  blocked. Thus, the invention provides for direct hybridization and primer-template formation between linear complements and single strand, circular template.

The invention also provides a process for generating multiple linear complements of a single strand, circular
25  nucleic acid template containing at least one cleavage site by: (a) combining the single strand, circular nucleic acid template with polynucleotide primers under conditions sufficient for hybridization; (b) extending the polynucleotide primer more than once around the circle to
30  generate a complementary displacement product comprising more than one contiguous complement of the single strand, circular nucleic acid template; (c) cleaving the displacement product to generate linear complements of the circle template; (d) combining the single strand, circular
35  template of step (a) with the linear complements produced in step (c) under conditions sufficient for hybridization;

(e) repeating steps (b) through (d) at least once to generate linear complements of the circle template.

By subsequent hybridization, or preferably simultaneous hybridization, of the initially produced linear complements, the products of more than one cycle (steps a through c above which correspond to Figure 2, steps G through J) of linear complements can be generated. Figure 2(K, parts 3-6) shows a schematic diagram depicting the hybridization of linear complements to single strand, circular templates and generation of additional displacement products and linear complements. Thus, repetitive cycles of priming, strand displacement synthesis, cleaving of the linear complements and repriming with linear complement exponentially amplifies the copy number of the initial polynucleotide primer. Generation of multiple complements of the circle template indicates the presence of the initial polynucleotide primer.

The invention also provides a process for generating multiple linear complements of a single strand, circular template where all steps are carried out under isothermal conditions. Using the preferred embodiments of the present invention, all reactions including, for example, hybridization of polynucleotide primers, restriction polynucleotides and linear complement, strand displacement synthesis and restriction endonuclease digestion of displacement products can be performed at the same temperature. Preferably this temperature is between about 25˚ and 70˚C, more preferably between about 37˚C to 50˚C. Isothermal conditions allow multiple and overlapping steps in the cycle to be performed rapidly and simultaneously.

Alternatively, the single strand, circular template can be denatured by heat prior to or during hybridization with linear complement. Heat denaturation ensures the availability of all templates for subsequent rounds of

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synthesis. Linear complements, once restricted, can also be denatured by heat prior to or during hybridization with circular template, ensuring the complete removal of the restriction polynucleotide for efficient primer-template
5 hybridization.

The invention utilizes synthetic polynucleotide primers as well as synthetic restriction primers. Chemical synthesis of polynucleotide primers is well known to those skilled in the art. Methods disclosed herein for the
10 synthesis of linear polynucleotides, such as phosphoramidite nucleotide chemistry, are routinely performed on automated synthesizers using protocols and reagents suggested by the manufacturers.

The invention further provides a process for
15 generating multiple linear complements of a circular template where the polynucleotide primers comprise a mixture of double and/or single-stranded nucleic acids. For example, the presence of a nucleic acid analyte within a biological sample can be determined by the processes
20 disclosed herein. The analyte can be a nucleic acid from an infectious organism such as a virus or bacteria. The analyte can also be nucleic acid derived from a host organism such as a human. The detection of nucleic acids derived from almost any source allows the invention to be
25 applicable in a large variety of clinical settings. Many infectious diseases and genetic disorders can be easily diagnosed using the invention.

Nucleic acids to be analyzed for such diseases and disorders can be isolated from the appropriate tissue or
30 body fluid of an organism and used as polynucleotide primers in the generation of linear complements. If the analyte is RNA, the appropriate polynucleotide primers and their 3' ends for polymerase extension can be generated by first and second strand cDNA synthesis using preselected

first or second strand primers. If the analyte is DNA, the
appropriate 3' ends can be generated for primer extension
by digesting the DNA with a preselected restriction enzyme.
Accordingly, any nucleic acid analyte can be used, or can
5  be generated using ordinary skills in the art, so long as
the 3' terminal nucleotides are sufficiently complementary
to the circular template to form a primer-template.

In the process of the present invention, any 3'-ends
of nucleic acids which are significantly complementary to
10 the single-stranded circular nucleic acid may initiate
synthesis of displacement products and linear complements.
If such nucleic acids are not the desired polynucleotide
primer, such as an analyte target sequence, generation of
non-specific background (false positive signal) may be
15 produced. To decrease any non-specific background, the
mixture of nucleic acids containing target sequences may be
treated with nucleic acids complementary to some portio: of
the target. Such complementary nucleic acids or catcner
nucleic acids may be immobilized on solid supports, such as
20 beads or membranes, or contain a ligand, for example,
biotin, which can be used to remove the hybridized catcher
and target sequences from solution. After removal of any
non-hybridized nucleic acids, the target fragment may now
be used to prime the single-stranded, circular nucleic
25 acid. Such affinity selection of target polynucleotide
primer greatly reduces non-specific backgrounds.

If double-stranded nucleic acids, such as DNA or cDNA,
are used or have been generated to use as polynucleotide
primers, the strands are separated by denaturation prior to
30 hybridization with the circular templates. Heat or
alkaline denaturation are two common procedures for the
separation of nucleic acid strands but a variety of other
methods are known to one skilled in the art which can also
be used. Detection of the analyte in the form of
35 intermediary displacement products or in the form of linear

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complements can be performed using standard fluorescent
staining or hybridization techniques known to one skilled
in the art. The invention also provides a process for
detecting multiple linear complements of a single strand,
5   circular nucleic acid template containing at least one
cleavage site by the steps of: (a) combining the single
strand, circular nucleic acid template with polynucleotide
primers under conditions sufficient for hybridization; b)
extending the polynucleotide primer more than once around
10  the circle to generate a complementary displacement product
comprising more than one contiguous complement of the
single strand, circular nucleic acid template; (c) cleaving
the displacement product to generate linear complements of
the circle template; (d) combining the single strand,
15  circular template of step (a) with the linear complements
produced in step (c) under conditions sufficient for
hybridization; (e) repeating steps (b) through (d) at least
once to generate linear complements of the circle template;
and (g) detecting the linear complements.

20      The invention further provides products such as DNA,
RNA, polypeptides and antibodies produced from either the
displacement products, displacement products hybridized to
restriction polynucleotides, linear complements or linear
complements hybridized to restriction polynucleotides.
25  These nucleic acids products, or nucleic acids encoding
these products, can be cloned into a vector for propagation
in either encaryotic or procaryotic organisms using
ordinary skills in the art. The choice of the vector will
depend on the organism in which the nucleic acid will be
30  propagated. Additionally, the cloned products can be
expressed _in vivo_ for polypeptide production (using
expression vectors) or they can be transcribed and (using
transcription vectors) and translated _in vitro_ to produce
recombinant polypeptides. Such recombinant polypeptides
35  derived by _in vivo_ or _in vitro_ methods can further be used
to generate monoclonal or polyclonal antibodies as

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described by Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988, which is incorporated herein by reference.

5        The following examples are intended to illustrate but not limit the invention.


                              EXAMPLES


        Circular M13mp18DNA (single or double stranded) was purchased from U.S. Biochemical (Cleveland, OH). All
10  synthetic oligonucleotides were made using standard phosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer (Applied Biosystems, Foster City CA). Purifications of DMT-on oligonucleotides was by "DMTon" reverse phase HPLC using 0.3 x 15 cm C-8 ($3\mu$) columns
15  eluting 5 to 30% acetonitrile in 100 mM triethyl-ammonium acetate at 1.0 ml/min. Phosphoramidites of 5-methyl-2' deoxycytidine and 5'- phosphorylating reagent were purchased from Glen Research, Herdon, VA. Where indicated, oligonucleotide linkers are synthesized with a blocked 3'-
20  end by synthesis on Branched Modifier Icaa-CPG (Glen Research), which results in oligomers with alkylamines on the 3'-hydroxyl. Size estimation of circles and extension products were based on mobility by agarose (0.5-4%) or polyacrylamide (4-20%) gel electrophoresis; size markers
25  were either Hae III restricted double-stranded Phix174 or Hae III-restricted single-stranded M13 DNA. Total nucleic acid synthesis was quantitated by ionic adsorption of nucleic acid onto DE-81 disks after incorporation of $^{32}$P-dATP or $^{32}$P-dCTP using the method as described in Molecular
30  Cloning: A Laboratory Manual, volume 3, 1988 Sambrook et al, eds, p. E18-E19. The number of primers extended in a reaction was quantitated by using $^{32}$P-end labeled primers of known specific activity.


        The average number of complements produced from a

primed single stranded DNA circle was determined as
follows:  the circular single stranded DNA is primed by
addition of a ten fold molar excess of complementary
primer.  A primer extension reaction was carried out on the
5   bound primer in the presence of alpha-$^{32}$P-dATP of known
specific activity.  The total amount of $^{32}$P incorporated
into circle complement was measured by spotting 2-5 $\mu$L of
the reaction mixture onto Whatman DE81 ion exchange paper.
The DE81 paper was washed five times in 0.3M NaPO$_4$ pH 7.0
10  for 5 minutes per wash to remove unincorporated $^{32}$P-dATP.
The DE81 paper was counted by scintillation counting.  The
number of adenosine residues per circle complement and the
circle concentration was known; allowing calculation of
specific activity per circle complement and the number of
15  circle complements produced per circle.


EXAMPLE I
Extension of a 7300 Base Pair M13
Single-Stranded Circle to Produce Multiple Copies


This example illustrates the ability of DNA
20  polymerases to displace complement strands produced after
extension and the ability of polymerases to incorporate
several hundred bases per minute.


A 19 $\mu$l reaction volume contained 50 nM M13 model
primer (sequence:    5'-dGGTTTTCCCAGTCACGACG) and 5 nM
25  M13mp18 single-stranded circular DNA (U.S. Biochemical,
Cleveland, OH) in Extension Buffer (10 mM Tris, pH 7.4, 10
mM MgCl, 1 mM dithiothreitol (DTT)) containing 200 $\mu$M dGTP,
dCTP, dTTP, and alpha-$^{32}$P-dATP (6 $\mu$Ci/$\mu$mol).  The extension
was initiated by addition of 1 $\mu$l containing 2 units of DNA
30  polymerase Klenow fragment (BRL, Gaithersburg, MD).
Negative controls consisted of either no primer added or no
enzyme added.  The extension was incubated for one hour at
either 37° or 50°C.  Total DNA synthesis was determined by
spotting 2 $\mu$l aliquots on DE81 anion exchange paper disks
35  (Whatman, Clifton, NJ), and washed three times in 0.5 M

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sodium phosphate, pH 7.3. Aliquots of 2-5 µl were also analyzed by agarose gel electrophoresis followed by autoradiography on XAR film (Kodak, Rochester, NY) to estimate average size of fragments produced by the

5  extension. The results indicated that the average size of circle complement produced was greater than 25,000 bases, or an extension rate of more than 417 bases per minute. This represents more than 18,000 bases of displacement.

EXAMPLE II

10
## Construction of a 682-Base
## Single-Stranded Circle Based on M13

This example illustrates the construction of a medium size single-stranded circle using recombinant enzymatic methods.

15  One milligram of single-stranded M13mp18 (U.S. Biochemical, Cleveland, OH) was ethanol precipitated from 1500 µl total volume containing 300 mM sodium acetate, pH 5.4, and pelleted by centrifugation. The supernatent was removed, and the pellet washed with 70% ethanol and dried

20  under vacuum. The DNA was redissolved in 500 µl water. A portion of the solution (90 µl, 180 µg of DNA = 76 pmols) was treated in the following manner: 1.5 µl (750 pmols) of each of two restriction site oligonucleotides were added (500 µM Bgl II/M13 restriction site oligonucleotide

25  sequence: 5'-dTGAGAGATCTACAAAGGCTATCAGGTCATTGCCT; AND 10 µM Bam H1/M13 restriction oligonucleotide, sequence 5'-dTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCG); 15 µl of 10x Universal buffer (Stratagene Cloning Systems, La Jolla, CA) plus 27 µl H₂0 was added. The mixture was heated to 65°C

30  for 3 minutes, then cooled on ice for 5 minutes. 7.5 µl of Bam HI restriction endonuclease (Boehringer Mannheim, Indianapolis, IN) and 7.5 µl of Bgl II restriction endonuclease (Boehringer Mannheim, Indianapolis, IN) was added and the mixture was incubated at 37°C for three

35  hours.

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After incubation, the digest was checked for completion on a 1.5% agarose mini-gel with approximately 0.5 µg of the digested DNA. A preparative 1.5% agarose (FMC SeaKem GTG, Rockland, ME)/TAE was prepared with marker
5    lanes. Agarose gel loading dyes (15.0 µl) were added to the digest. Ten µl of this preparation were loaded in the marker lane and the remainder in the preparative well. The sample was electrophoresed in TAE buffer for 1.5 hours at 25 mA.

10    The marker lane was cut from the gel and stained with ethidium bromide in water at a concentration of 0.5 µg/ml. The 682 bp fragment was located under UV light and then excised from the corresponding location on the gel.

The gel slice was loaded onto a Schleicher & Schuell
15    Elutrap (Schleicher & Schuell, Keene, NH) and electroeluted in 1x TAE buffer for 4 hours at 130 V. The eluate was recovered and butanol extracted 4 times, Phenol/chloroform/isoamyl alcohol extracted twice and chloroform/isoamyl alcohol extracted one time. One tenth
20    volume of 3 M sodium acetate pH 5.4 and 2.5 volumes of 100% ethanol were added and the preparation was incubated in an ethanol/dry ice bath for 20 minutes. The preparation was then centrifuged in a microfuge for 30 minutes at room temperature at 14,000 X G. The supernate was discarded,
25    0.75 ml of 70% ethanol was added to wash the pellet and the preparation was then re-centrifuged for 5 minutes under the same conditions. Ethanol was removed and the pellet was dried under vacuum for 3 minutes.

The dried pellet was redissolved in 100 µl of H₂0 and
30    loaded onto a Centricon 100 (Amicon, Danvers, ME). Two mls of 50 mM NaOH/1 mM EDTA were added and the preparation was spun in a fixed angle rotor, (Model SS34; Dupont-Sorvall, Wilmington, DE) centrifuged for 45 minutes at room

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temperature at 2400 rpm.  The above steps were repeated with an additional two mls of NaOH/EDTA.  One ml of TE 10:1 pH 8.0 (10 mM Tris pH 8.0, 1 mM EDTA) was added and centrifuged as above.

5      Circularization of the 682 bp fragment was performed as follows:  The retentate was recovered from Centricon 100 filter apparatus at a total volume of 38 μl.  Forty μl of 5X ligase buffer (BRL, Gaithersburg, MD) were added.  A linker molecule to span both ends of the linear 682 bp
10  fragment was synthesized as described above.  The synthesized linker molecule, designated M13/682 ligation linker, had the following sequence: 5'dCGACTCTAGAGGATCTACAAAGGCTATCAGCAA.  One hundred and seven μl of H₂0 were added and the preparation was incubated
15  at 65°C for 5 minutes, followed by flash cooling on ice for 5 minutes.  Three μl of T4 DNA Ligase (BRL, Gaithersburg, MD) containing 1U/μl were added and the preparation was incubated at 16°C for 1 hour.  Following incubation, two additional μls of linker were added.  The preparation was
20  incubated at 65°C for 5 minutes, cooled on ice 5 minutes, and then three μls of ligase were added followed by a 1 hour incubation at 16°C.  Two additional μls of linker were added, followed by another 65°C incubation, rapid cooling on ice for 5 minutes.  Three more μls of ligase were added
25  and reaction was incubated at 16°C overnight.


EXAMPLE III

Construction of a Synthetic 72-Base
Single-Stranded Circle with Protected
Restriction Sites and a Single Core Sequence

30-     This example illustrates the construction of a small single-stranded circle using oligonucleotide synthesis followed by enzymatic ligation to form the circle.

A 72 base DNA oligomer with a 5'-phosphate ( sequence: 5'-p-d   CTTTCACGTTTATCATCTGACTATCCTGTAATAAAGATCAATGCGT*C*C

GGTCTACATAGTCTCTAAAATTAG) was synthesized on an Applied
Biosystems 380A DNA synthesizer (Applied Biosystems, Foster
City, CA) using standard phosphoramidite chemistry. Both
deoxycytidines in the HpaII restriction site (CCGG)
5    contained within the 72 base DNA oligomer were replaced
with 5-methyldeoxycytidine to prevent cleavage of the
oligomer by Hpa II. A 33 base linker sequence that has 15
bases complementary to each end of the 72 base oligomer was
also synthesized. The 3' end of the linker has 3
10   noncomplementary bases and a blocked 3' end to prevent the
linker from extending in a polymerase reaction. This
oligomer was used to bring the 5' and 3' ends of the 72
base oligomer together to allow for enzymatic ligation.

The 72 base oligomer was enzymatically ligated in a
15   1mL reaction volume containing 10uM of 72 base oligomer,
5uM of 33 base linker oligomer, 40mM Tris-Cl pH 7.5, 10mM
$MgCl_2$, 10mM dithiothreotol, and 1mM ATP. The reaction
mixture was incubated for 5 minutes at 50°C then cooled to
room temperature. 40,000 units of T4 DNA Ligase (New
20   England Biolabs, Beverly, MA) was added to the reaction
mixture. The reaction mixture was incubated for 2 hours at
room temperature. The linker concentration was brought to
10uM and the reaction mixture was incubated an additional
16 hours at room temperature. The linker concentration was
25   increased to 15uM and the reaction mixture was incubated an
additional 2 hours at room temperature. The reaction
mixture was electrophoresed at 1000V for 4 hours in a 10%
polyacylamide/8M urea gel in 1xTBE. The slower migrating
circular 72mer product was identified by UV shadowing,
30   electroeluted, ethanol precipitated and quantitated by
optical density at 260nm. The circular 72mer was resistant
to degradation by Exo VII, and could be primer extended
past the ligation site.

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## EXAMPLE IV

### Construction of a Synthetic 101-Base Single-Stranded Circle with Protected Restriction Sites and a Single Core Sequence

5      A synthetic 101mer oligomer (sequence: 5'- pd<u>GATTAAAATCTCTGA</u>CCCGAGCCGATGACTTACTGGC<sup>m</sup>CG<sup>m</sup>CGTT<sup>m</sup>CGAA<sup>m</sup>C<sup>m</sup>CGGA AAGCTCCCTGGAGTGCGATCTTCCTGAGGCCGAT<u>CTGATAGGACATTAT</u>) was synthesized as in Example III. The underlined portion designates the sequence which is complementary to the 33
10 base linker described below. The 101mer oligomer contained blocked restriction sites for the restriction endonucleases Hpa II (CCGG), Bst B1 (TTCGAA), and Bst U1 (CGCG). All the deoxycytosines in the restriction sites of these restriction endonucleases were replaced with 5-
15 methyldeoxycytosines to prevent restriction cleavage. A 33 b a s e  l i n k e r  ( s e q u e n c e :  5 ' - dTCAGAGATTTTAATCATAATGTCCTATCAGTAG) that has 15 bases complementary to each end of the 101 base oligomer was also synthesized. The 3' end of the linker has 3
20 noncomplementary bases and a 3' amine to prevent the linker from extending in a polymerase reaction. This oligomer was used to bring the 5' and 3' ends of the 101 base oligomer together to allow for enzymatic ligation. The 101 base oligomer was enzymatically ligated and purified as in
25 Example III. The circular 101mer was resistant to degradation by Exo VII and could be primer extended past the site of ligation.

## EXAMPLE V

### Construction of a Synthetic 202-Base Single-Stranded Circle with Protected Restriction Sites and a Repeated Core Sequence
30

This example illustrates the construction of a small single-stranded circle composed of two core sequences with unique ends enzymatically ligated to form the circle.

35      A synthetic 101 base oligomer (sequence: 5'-

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pd<u>TTCTAGTTACGCAGG</u>CCCGAGCCGATGACTTACTGGCCGCGTTCGAACCGGAAAG
CTGGCTGGAGTGCGATCTTCCTGAGGCCGA<u>TACTATTTGCACTTTC</u>)        was
synthesized as in Example III with the same 71 base core
sequence but with different 15 base linker sequences on
5    each end of the 101 base oligomer.  The different linker
sequences,  which  are  underlined,  allow  for  specific
ligation between the 101 base oligomer shown above and the
101 base oligomer from Example IV.  Two 34 base linkers
(sequences:   (1)   5'-dCCTGCGTAACTAGAAATAATGTCCTATCAGTTTT-
10   $NH_2$ and  (2)      5'-dTCAGAGATTTTAATCGAAAGTGCAAATAGTCCCC-$NH_2$)
that have 15 bases complementary to one end of each 101
base oligomer were also synthesized.  The 3' end of the
linkers have 4 noncomplementary bases and a 3' amine to
prevent  the  linkers  from  extending  in  a  polymerase
15   reaction.  Each linker wwas used to bring the 5' end of one
101 base oligomer and 3' end of the other 101 base oligomer
together to allow for enzymatic ligation.

     The 101 base oligomers were enzymatically ligated and
purified into a 202 base oligomer as in Example III except
20   that each 101 base oligomer was at 0.6 $\mu M$ and the first
linker was at 0.15 $\mu M$ starting concentration and increased
to 0.75 $\mu M$.  The 101 base oligomers were also gel purified
before ligation instead of after ligation.  The 202 base
oligomer was then ligated into a circle as in Example III
25   except the 202 base oligomer was at 0.3 $\mu M$ and the second
linker was at 0.15 $\mu M$ starting concentration and increased
to 1.2 $\mu M$.


EXAMPLE VI

Extension of a 682-Base Single-Stranded Circle


30       This  example  illustrates  the  ability  of  DNA
polymerases to displace complement strands produced after
extension, and the ability of polymerases to incorporate
several hundred bases per minute into a medium-sized
single-stranded circle.  A primer extension reaction was

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carried out and extension products determined as described in Example I. Extension reactions for the circular 682 bp single strand, nucleic acid were performed using free nucleotides, a DNA Polymerase (BRL, Gaithersburg, MD), a
5   synthetic $^{32}$P labeled primer fragment (5000 cpm/fmole) and T4 Polynucleotide Kinase (New England Biolabs, Beverly, M A ) . The primer sequence was 5'TGAACGGTAATCGTAAAACTAGCATGTCAATCATATGTACCCCGGTTGAT3'. Reactions were performed in 20 µl volumes with a 10:1 molar
10  ratio of primer to circle, dithiothreotol (DTT) at a final concentration of 5 mM, free dNTP's (Pharmacia, Pleasant Hill, CA) at a final concentration of 200 µM, Klenow DNA polymerase (Boehringer Mannheim, Indianapolis, IN), and 10X extension buffer. Reactions were incubated at 37°C for one
15  hour. Three and a half µls of 6X alkaline gel loading buffer were added to the reactions and then electrophoresis was carried out in a 0.7% alkaline agarose gel for 16 hours at 150 mA. The gel was neutralized and stained as described above and then exposed on Kodak X-OMAT AR film
20  (Kodak, Rochester, NY) at room temperature with a screen for two hours to analyze extension products. The average size of circle complement produced using a 682-base single-stranded circle was greater than 20,000 bases.

EXAMPLE VII
25      Extension of a 72-Base Circle

A primer extension reaction was carried out as in Example I except a different primer was used (sequence: 5'-dACAAGGACCAAAAGAACCTTTTAGAGACTATGTAGAC). The 19 bases on the 3' end are complementary to the 72 base circle but
30  the 18 bases on the 5' end are not complementary to the circle. The average size of circle complement produced using a 72-base single-stranded circle was 150 to 250 bases.

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## EXAMPLE VIII

### Extension of a 7300-Base Single-Stranded Circle, and Restriction to Form Secondary Primers

This example illustrates the ability of Sau 3A
5  endonuclease to restrict the amplified product of a 7300-
base single-stranded circle to generate linear complements
for use as secondary primers in the next cycle of extension
reaction.

A 19 $\mu$l reaction volume contained 50 nM M13mp18
10  single-stranded circle (U.S. Biochemical, Cleveland, OH)
and 5 nM M13 primer (100 fmole) in extension buffer (20 mM
Tris pH 7.5, 10 mM MgCl$_2$, 25 mM NaCl, 6.5 mM DTT) containing
200 $\mu$M dGTP, dCTP, dATP, dTTP and 0.33 $\mu$M of alpha $^{32}$P-
dATP.  The final specific activity of $^{32}$P-dATP was 9,000
15  cpm/pmole.  The extension was initiated by addition of 1 $\mu$l
of DNA polymerase Klenow fragment (BRL, Gaithersburg, MD,
6 units/$\mu$l) for 1 hour at 50°C.  2 $\mu$l of 8 units/$\mu$l Sau3A
I enzyme (BRL, Gaithersburg, MD) were added to the 20 $\mu$l
reaction mix and allowed to digest for 1 hour at 37°C.  4
20  $\mu$l of the 20 $\mu$l reaction mix was transferred into a new
tube and denatured for 2 minutes at 100°C in a heat block
(silicon oil/sand or boiling water), followed by chilling
in a dry ice bath for 1 minute.  16 $\mu$l of new reaction mix
containing 40 nM M13mp18 single-stranded circle and the
25  same concentration of extension buffer mentioned above was
added to the 4 $\mu$l chilled sample.  1 $\mu$l of DNA polymerase
Klenow fragment was added and the extension reaction was
carried   out   for   1   hour   at   50°C.      The
restriction/denaturation/extension cycles were repeated
30  two more times.  The restricted and extended products were
analyzed by gel and total DNA synthesis.  2 $\mu$l of each
sample tube was loaded onto a 0.5% alkaline agarose
denaturing gel and run for overnight at 150 mA.  A 1:10
diluted stock of each sample tube was prepared and 2 $\mu$l was
35  used to spot onto DE81 Whatman paper, washed 3 times in

0.5M sodium phosphate, pH 7.3 and counted in a scintillation counter for total count analysis. Denatured, extended samples were used as controls. Results indicated that the extended product was restricted and the restricted

5  fragments in turn acted as primers for the next round of extension.  Since each molecule of M13mp18, (complement contains about 1760 adenine residues, incorporation of 9,000cpm/pmol $^{32}$p-dATP will result in complement product with specific activity of 16,000 cpm/fmol.  Results

10 (E=extension; R=restriction; D=heat denaturation)

| Reaction | cpm incorporated | fmols complement | fold increase |
|---|---|---|---|
| E only | $6.4 \times 10^6$ | 400 | 4 |
| E/R | $2 \times 10^7$ | 1,250 | 12.5 |
| E/R/D/E | $2 \times 10^8$ | 12,500 | 125 |
| E/R/D/E/R | $3 \times 10^8$ | 18,750 | 187.5 |
| E/R/D/E/R/D/E | $2.2 \times 10^9$ | 137,500 | 1,375 |

These results indicate that 1,375 molecules of circle

20 complement are formed for every original molecule of polynucleotide primer, or a 1,375 fold amplification over original target polynucleotide.  Repeating the reaction under isothermal conditions at 50°C gave similar results.


EXAMPLE IX

25  Extension of a 72-Base Single-Stranded
Circle, and Restriction to Form Secondary Primers


A primer extension reaction was carried out as in Example VII except Taq DNA Polymerase (Cetus, Emeryville, CA) was used at 60°C instead of Klenow at 37°C.  The

30 extended product was brought to 5 mM in DTT and 1 μM in a 38 base restriction linker (sequence:      5'-dTGACTATCCTGTAATAAAGATCAATGCGTCCGGTCTAC-NH$_2$).      The restriction linker is complementary to the circle complement and renders it double-stranded.  10 units of Hpa

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II restriction endonuclease were added and the reaction mix was incubated 30 minutes at 37°C. The product was visualized by autoradiography. The circle complement was cleaved into 72 base fragments following this procedure.

5       Although the invention has been described with references to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims.

WE CLAIM:

       1.    A process for generating multiple linear complements of a single strand, circular nucleic acid template containing at least one cleavage site comprising:

5

    (a)   combining the single strand, circular nucleic acid template with polynucleotide primers under conditions sufficient for hybridization; and

10

    (b)   extending the polynucleotide primer more than once around the circle to generate a complementary displacement product comprising more than one contiguous complement of the single strand, circular nucleic acid template.

2. A process for generating multiple linear complements of a single strand, circular nucleic acid template containing at least one cleavage site comprising:

(a) combining the single strand, circular nucleic acid template with polynucleotide primer under conditions sufficient for hybridization;

(b) extending the polynucleotide primer more than once around the circle to generate a complementary displacement product comprising more than one contiguous complement of the single strand, circular nucleic acid template;

(c) cleaving the displacement product to generate linear complements of the circle template;

(d) combining the single strand, circular template of step (a) with the linear complements produced in step (c) under conditions sufficient for hybridization; and

(e) repeating steps (b) through (d) at least once to generate linear complements of the circle template wherein the last repeated cycle ends at step (b) or (c).

3. The process of claim 2, wherein the circular template of step (a) is denatured prior to use in step (d).

4.    The process of claim 2, wherein the product of step (c) is denatured prior to use in step (d).

5.    The process of claim 1 or 2, wherein all steps are carried out under isothermal conditions.

6.    The process of claim 1 or 2, wherein the polynucleotide primers comprise double-stranded nucleic acids, single-stranded nucleic acids or a mixture thereof.

7.    The process of claim 6, wherein the nucleic acids are derived from a biological sample.

8.    The process of claim 6, wherein the polynucleotide primers are double-stranded and their strands are separated by denaturing before or during step (a).

9.    The process of claim 8, wherein the denaturing is thermal.

10.    The process of claim 1 or 2, wherein the polynucleotide primers are synthetic oligonucleotides.

11.    The process of claim 1 - 2, wherein the polynucleotide primers are selected so as to allow strand displacement synthesis by a polymerase.

12.    The process of claim 1 or 2, wherein the 5' end of the polynucleotide primers are non-complementary to the circular template.

13.    The process of claim 1 or 2, wherein the 5' end of the nucleotide primers are blocked.

14. A process of synthesizing a single strand, circular nucleic acid comprising:

      (a) chemically synthesizing a linear polynucleotide;

5      (b) combining the linear polynucleotide with a complementary linking oligonucleotide under conditions sufficient for hybridization; and

      (c) ligating the linear polynucleotide to
10      produce a single strand, circular nucleic acid.

15. The process of claim 14, wherein the circular nucleic acid is synthesized as two or more linear polynucleotides.

16. A single strand, circular nucleic acid, wherein the nucleic acid comprises between 30 and 2200 nucleotides.

17. The single strand, circular nucleic acid of claim 16, wherein the nucleic acid comprises between 30 and 750 nucleotides.

18. The single strand, circular nucleic acid of claim 17, wherein the nucleic acid comprises between 50 and 300 nucleotides.

19. The single strand, circular nucleic acid of claim 18, wherein the circular nucleic acid further comprises at least one cleavage site.

20. The single strand, circular nucleic acid of claim 19, wherein a portion of the circular nucleic acid is complementary to polynucleotide primers derived from a biological sample comprising double-stranded nucleic acids,
5　single-stranded nucleic acids or a mixture thereof.

21. The process of claim 1 or 2, wherein the single strand, circular nucleic acid template is derived from phage replication.

22. The process of claim 1 or 2, wherein the single strand, circular nucleic acid template is between 50 and 2200 nucleotides.

23. The process of claim 1 or 2, wherein the single strand, circular nucleic acid template is made from chemically synthesized oligo- or poly-nucleotides.

24. The process of claim 23, wherein the single strand, circular nucleic acid template is between 50 and 750 nucleotides.

25. The process of claim 1 or 2, wherein the cleaving of the displacement product is achieved by one or more restriction endonucleases.

26. The process of claim 25, wherein the one or more restriction endonuclease sites on the single strand, circular nucleic acid template are capable of being modified so as to inhibit restriction of at least the
5　modified strand.

27. The process of claim 25, wherein the restriction endonuclease site is Hpa II.

28.  The process of claim 26, wherein the one or more restriction endonuclease sites are modified by methylation.

29.  The process of claim 1 or 2, wherein the restriction polynucleotides are selected so that when restricted, a fragment of the restriction polynucleotide remains hybridized to the 5' end of the displacement product.

30.  The process of claim 1 or 2, wherein the restriction polynucleotides are deoxyribonucleic acids and the 3' end is inhibitory to primer extension by a polymerase.

31.  The process of claim 1 or 2, wherein the polymerase is selected from the group comprising E. coli DNA polymerase I, Klenow fragment, T4 DNA polymerase, T7 DNA polymerase, modified polymerases, reverse transcriptase, Taq polymerase, Bst polymerases or T. flavins polymerase.

43

32.   A process for detecting multiple linear
complements of a single strand, circular nucleic acid
template containing at least one cleavage site comprising:

5
(a)   combining the single strand, circular
nucleic acid template with
polynucleotide primers under conditions
sufficient for hybridization;

(b)   extending the polynucleotide primer
more than once around the circle to
10
generate a complementary displacement
product comprising more than one
contiguous complement of the single
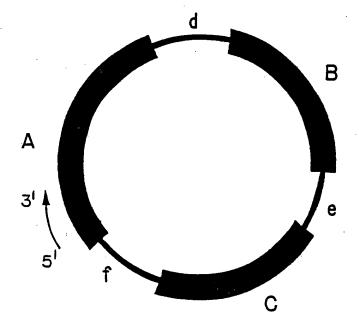strand, circular nucleic acid template;
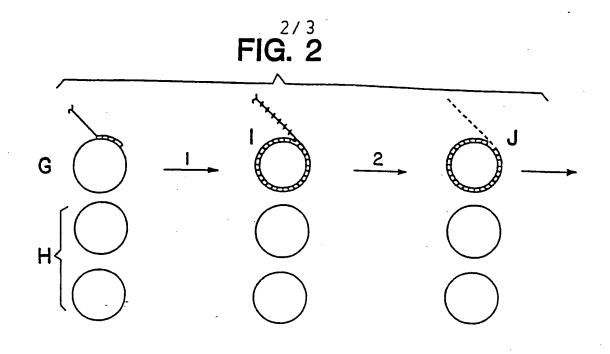
(c)   detecting the linear complements.

33.   A process for detecting multiple linear complements of a single strand, circular nucleic acid template containing at least one cleavage site comprising:

5

(a)   combining the single strand, circular nucleic acid template with polynucleotide primers under conditions sufficient for hybridization;

(b)   extending the polynucleotide primer more than once around the circle to generate a complementary displacement product comprising more than one contiguous complement of the single strand, circular nucleic acid template;

10

(c)   cleaving the displacement product to generate linear complements of the circle template;

15

(d)   combining the single strand, circular template of step (a) with the linear complements produced in step (c) under conditions sufficient for hybridization;

20

(e)   repeating steps (b) through (d) at least once to generate linear complements of the circle template, wherein the last repeated step ends at step (b) or (c); and

25

(f)   detecting the linear complements.

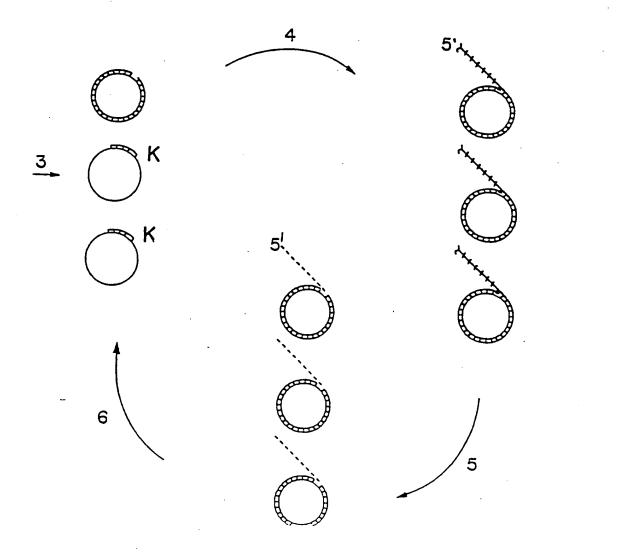34.   The process of claim 32 or 33, wherein the detecting step is by hybridization.

45

35.   The process of claim 32 or 33, wherein the detecting step is by fluorescence.

36.   The process of claim 32 or 33, wherein the polynucleotide primers comprise double-stranded nucleic acids, single-stranded nucleic acids or a mixture thereof.

37.   The process of claim 36, wherein the nucleic acids are derived from a biological sample.

38.   The displacement products produced by the process of claim 1 or 2.

39.   The displacement products hybridized to the restriction polynucleotides produced by the process of claim 1 or 2.

40.   The linear complements produced by the process of claim 1 or 2.

41.   A product made with the product of claim 38 selected from the group consisting of DNA, RNA, polypeptide and antibody.

42.   A product made with the product of claim 39 selected from the group consisting of DNA, RNA, polypeptide and antibody.

43.   A product made with the product of claim 40 selected from the group consisting of DNA, RNA, polypeptide and antibody.

44.   The process of claim 1 further comprising step (c) cleaving of the displacement product to generate linear complements of the circle template.
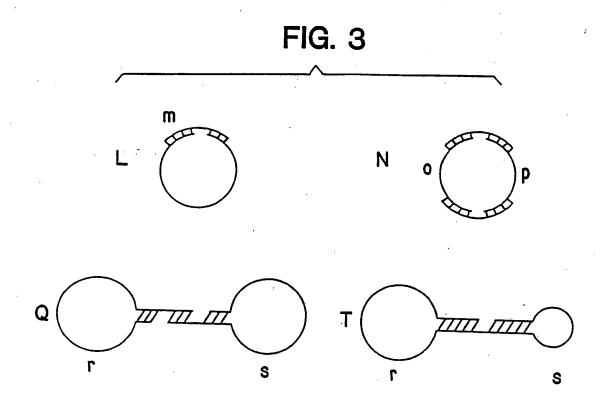
45.  The process of claim 1 or 2, wherein the polynucleotide primers are affinity selected.
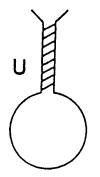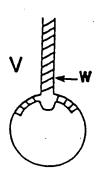
# FIG. 1

# FIG. 2

# FIG. 3

# INTERNATIONAL SEARCH REPORT

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68; C12P 19/34
U.S. CL.: 435/6, 91; 436/501; 935/16, 17, 78

## II. FIELDS SEARCHED

| Minimum Documentation Searched 7 | |
|---|---|
| Classification System | Classification Symbols |
| U.S. CL. | 435/6, 91; 436/501; 935/16, 17, 78 |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

APS and DIALOG databases were s..rch

## III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

| Category* | Citation of Document, 11 with indication, where appropriate, of the relevant passages 12 | Relevant to Claim No. 13 |
|---|---|---|
| Y | Watson, "Molecular Biology of the Gene", 3rd rD.. published N.W. Benjamin, Inc.. p. 238-241. See entire document. | 1-45 |
| Y | BIOSIS Abstract No. 69057578. issued 1979, "Bacteria Phage.... Microscopy" see abstract. J. Mol. Biol. 135(1) 69-90, KEEGSTRA ET AL, see the abstract. | 1-45 |
| Y | BIOSIS Abstract. No. 70030184, issued 1980, "The Rolling Circle...Assembly", see abstract. KOTHS et al.. J. Biol. Chem. 255(9), 4328-4338. | 1-45 |
| Y | BIOSIS Abstract No. 80067350. issued 1985, "The complete 30-Base-Pair... Packaging", see abstract, Eur. J. Biochem. 149 (3), 579-584, FLUIT et al. | 1-45 |

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 27 November 1991 | 16 DEC 1991 |
| International Searching Authority | Signature of Authorized Officer |
| ISA/US | Amelia B. Yarbrough      ebw |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|---|---|---|
| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
| Y | MEDLINE Abstract No. 87137459, issued 1987, MOK et al , "Formulation of rolling circle.... replication" see abstract, J. Biol. Chem., 262(5), 2304-2309. | 1-45 |
| Y | Nucleic Acids Research, Vol. 18, No. 9 issued 24 May 1990, CAPOBIANCO ET AL., "One pot solution synthesis of cyclic oligonucleotides", p. 2661-2669. See "cyclization reaction". | 14-15 |
| . Y | Journal of Bacteriology, vol. 171, No. 6, issued June 1989, SHAVITT ET AL. "Rolling-Circlee Replication of UV-Irradiated Duplex DNA in the X174 Replicative-Form Singl--Strand System in Vitro", p. 3530-3533, see entire document. | 1-45 |